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Metallocoenzyme-Mediated Reductive Transformation of Carbon Tetrachloride in Titanium(III) Citrate Aqueous Solution

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Transformation pathways for carbon tetrachloride (CCl_4) catalyzed by hematin or vitamin B_{12} in aqueous titanium(III) citrate solution are proposed. The reaction of CCl_4 with B_{12} was zero order in CCl_4 and first order in B_{12}, and the rate constant was measured from pH 7.3 to pH 10.3. The proposed rate-limiting step is the reduction of the stable trichloromethylcobalamin (CCl_3-Cbl) intermediate by titanium(III) citrate at alkaline pH and the sterically induced CCl_3-Cbl decomposition at neutral pH. The reaction kinetics can be described by a modified Michaelis–Menten model in the saturated regime. With hematin, only the pseudo-first-order rate constant was determined due to the significant deactivation of the coenzyme. The turnover number of hematin (molecules of CCl_4 transformed/molecule of hematin deactivated) was 27 at pH 8.0 and 42 at pH 9.9. Vitamin B_{12} was a more stable and more effective catalyst (on a molar basis) than hematin with respect to CCl_4. Chloroform ($CHCl_3$) was the primary product in titanium(III) citrate solution, and the yield was a function of pH, Ti(III) concentration, and organic content regardless of whether a coenzyme was present or which coenzyme was used. Although B_{12} and hematin can both enhance the CCl_4 transformation rate, they have little effect on the $CHCl_3$ yield. Titanium(III) citrate, on the other hand, controls not only the transformation rate but also $CHCl_3$ formation.

Introduction

The fate of anthropogenic chlorinated hydrocarbons in the aqueous and terrestrial environment is of great concern because of their potential toxicity and/or carcinogenicity. Mono- and dichlorinated compounds tend to transform via nucleophilic substitution or oxidation, whereas heavily chlorinated hydrocarbons are more susceptible to reductive transformation. CCl_4, for example, has been shown to degrade biologically under denitrifying (*1*, *2*), sulfate-reducing (*3*), and methanogenic (*4*) conditions and chemically by hydroquinone-type compounds and humic acid (*5*), iron–sulfur minerals (*6*), zero-valent metals (*7*), transition-metal cations (*8*, *9*), and metallocoenzymes (*10*).

Reductive dechlorination reactions involving coenzymes are of particular interest because they are presumably responsible in biological detoxification and activation processes. Many mammalian and bacterial enzymes contain as prosthetic groups a transition-metal coenzyme, such as heme (an iron porphyrin complex), cobalamins (vitamin B_{12} derivatives), and cofactor F_{430} (a nickel-centered porphinoid unique to methanogenic microorganisms). Heme is commonly found in aerobic cells and is the active site of various cytochromes. Cytochrome P_{450}, for example, can either detoxify or activate toxins entering the mammalian liver. Heme and hemoproteins have also been shown to reductively dehalogenate many organic halides *in vitro* (*10–18*). Cobalamins are abundant in anaerobic microorganisms. These cobalt complexes control various C_1 metabolic reactions, such as the methyl transfer reaction in methionine biosynthesis (*19–22*) and possibly microbial methane formation (*23*). Cobalamins and F_{430} are presumably the active components responsible for the dehalogenation reactions by methanogenic organisms (*24–27*). *In vitro*, they also function as electron carriers for catalytic reduction of organic halides in the presence of an external electron source (*10*, *16*, *24–29*). The Fe in heme commonly exists in di- or trivalent states, whereas the Co(III) in cyanocobalamin (also known as vitamin B_{12}) can be reduced to Co(II) (called B_{12r}) or Co(I) (B_{12s}), depending on the redox environment.

Reaction mechanisms and kinetic models describing reductive dehalogenation reactions involving metallocoenzymes are important for understanding and predicting biological effects of these chlorinated pollutants and for the potential application of the coenzyme system in detoxification processes (*14*). To date, although the transformation of haloorganic compounds and some coenzymes have been studied extensively (*11*, *19*, *30*, *31*), only limited kinetic and mechanistic data are available for the evaluation of these coenzymatic reactions in the presence of a bulk reducing agent in aqueous medium. The reactions catalyzed by porphyrin-type redox mediators have been described as a two-step electron transfer process (*32*, *33*): electrons are transferred from the bulk reductant to the catalyst, which in turn reduces the chlorinated substrate. However, experiments conducted in our laboratory using titanium(III) citrate or thio compounds as the reductant have shown that the actual mechanism may be more intricate than this model describes, and the reducing

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agent may control not only the catalysis rate but also subsequent reaction steps and, therefore, the product formation. Krone et al. (*25*) proposed several possible reaction schemes to describe the reduction of CCl_4 to methane by corrinoids in titanium(III) citrate solution, but the rate-limiting step was not identified. Wolf et al. (*34*) proposed a model system for the reduction of CCl_4 by the hemoprotein cytochrome P_{450}, but the results were questioned by Castro et al. (*35*). These authors reported different product distributions and hypothesized different reaction schemes. These observations prompted us to investigate the reaction mechanisms and the kinetics of the reductive dechlorination reactions mediated by cobalamin (vitamin B_{12}) or iron porphyrin (represented by hematin) in titanium(III) citrate solution. In this paper, we identify the rate-limiting step and propose a kinetic model for B_{12}-mediated CCl_4 transformation. We also hypothesize reaction mechanisms for both coenzyme systems, with special focuses of the study on the role of titanium(III) citrate and the relative catalytic efficiency and stability of these cofactors.

Citrate is a complexing agent added to prevent precipitation of titanium(III) hydroxide (*36*). The reducing power of titanium(III) citrate increases with pH: E° (vs SHE) evaluated by Zehnder (*37*) decreases linearly from −0.48 V at pH 7 to nearly −0.70 V at pH 10.

CCl_4 was selected as the model substrate in our studies because of its adverse environmental and health effects (e.g., hepatotoxicity (*38*)) and its relatively high reactivity. In addition, CCl_4 contains four identical C−Cl bonds and thus makes a good model substrate for chloroalkanes.

Materials and Methods

Chemicals. Carbon tetrachloride (>99%) and chloroform (99%) were purchased from Aldrich (Milwaukee, WI) and J. T. Baker (Phillipsburg, NJ), respectively. CCl_4 and $CHCl_3$ calibration standards were prepared in methanol and stored in borosilicate amber vials sealed with PTFE/silicone septa-lined screw-top caps at −10 °C when not in use. Deoxygenated Milli-Q water (Millipore, Bedford, MA) was saturated with CCl_4 and used as the spike solution. Titanium(III) citrate stock solutions were prepared from 1.9 M $TiCl_3$ in 2 M hydrochloric acid (Aldrich) following procedures similar to those described by Zehnder and Wuhrmann (*36*). The ratio of titanium(III):citrate was 1:2.6 in the final solution. Cyanocob(III)alamin (vitamin B_{12}, 99%), dicyanocobinamide (a structural analogue of cobalamin except lacking the 5,6-dimethylbenzimidazole group, 95%), and hematin (ferriprotoporphyrin IX hydroxide) were purchased from Sigma (St. Louis, MO) and used as received. A small amount of sodium carbonate was added to facilitate the dissolution of hematin in water (*13*). Stock solutions of these coenzymes were prepared in deoxygenated Milli-Q water and stored in an anaerobic glovebox (Coy, Ann Arbor, MI) in the absence of light. The glovebox had an atmosphere consisting of 90% N_2 and 10% H_2, and the anaerobic condition was maintained by palladium catalyst and indicated by reduced Safranin-O solution. Cob(II)alamin (B_{12r}) solution was prepared in the glovebox by the addition of 10 equiv of the dithiothreitol (DTT, >99%, Fluka, Ronkonkoma, NY) to 100 μM vitamin B_{12}. Thio reducing agents are known to reduce B_{12} to B_{12r} (*29, 39, 40*). The complete reduction of B_{12} to B_{12r} was indicated by the color change from pink to yellow and was also confirmed by UV−visible spectra.

Kinetic Study. Kinetic experiments were conducted at 22 ± 0.6 °C using 2-oz. borosilicate amber bottles capped with leak-tested screw-top Mininert valves (Alltech, Deerfield, IL). All bottles were washed with 10% nitric acid and thoroughly rinsed with deionized water prior to autoclaving. Deoxygenated Milli-Q water, prepared by sparging with 99.99% N_2 for 1 h, was placed immediately in the glovebox along with all other materials at least 24 h prior to use. Complete deoxygenation of the water was verified by the colorless (reduced) Safranin-O solution at pH 9 ($E^\circ = -320$ mV) before the water was used for the preparation of all reagent solutions in the glovebox. A 60-mL sample of aqueous reagent mixture, which contained a pH buffer (0.2 M), titanium(III) citrate (0.6−5.0 mM), and a catalytic amount of either hematin (up to 15 nM) or vitamin B_{12} (up to 1.0 nM), was placed in an amber bottle capped with a Mininert valve, leaving approximately 4 mL of head space. The actual volume of each individual bottle was measured by weight for concentration calculations. Due to the high volatility of CCl_4, the valve−bottle juncture was further tightly wrapped with low oxygen permeability vinyl tape (3M, St. Paul, MN) to obtain a better seal before the bottle was removed from the glovebox. Less than 5% of CCl_4 was lost from the control bottle due to volatilization over a period of 2 days.

The reaction was triggered by spiking saturated solution of CCl_4 in deoxygenated Milli-Q water into the reagent mixture to give the desired initial concentration (0.2−1.0 μM). The bottle was shaken constantly during the reaction at 400 rpm in an Orbit shaker (Lab-Line Instruments, Melrose Park, IL). Head space samples were withdrawn after predetermined times using a 250-μL gas-tight side-port syringe (Alltech). CCl_4 and $CHCl_3$ were monitored over at least two half-lives. A Hewlett-Packard (HP) Series II 5890 (Palo Alto, CA) gas chromatograph (GC) equipped with a ^{63}Ni electron capture detector (ECD) and a 15-m DB-1 fused silica capillary column (1.5 μm film thickness, J & W Scientific, Rancho Cordova, CA) was used. Analyses were isothermal at 35 °C, with helium and Ar/CH_4 as the carrier and the auxiliary gases, respectively. Peak areas were integrated with a PE Nelson 2600 Chromatography Data System (PE Nelson, Cupertino, CA). The aqueous concentrations of CCl_4 and $CHCl_3$ were calculated using calibration standards described above and the Henry's constants reported by Gossett (*41*). Statistically identical kinetic results were obtained when aqueous samples were taken instead, and analytes were extracted with pentane. An EA 940 expandable ion analyzer (Orion Research, Boston, MA) was used to measure the pH of the reagent solutions.

Using a similar experimental setup, we have estimated the turnover number in the hematin deactivation process during reaction with CCl_4 either by sequentially respiking CCl_4 and measuring the apparent pseudo-first-order rate constant (k_1') or by adding a limited amount of hematin and monitoring the change in k_1' upon complete deactivation of the cofactor.

Evaluating Zero-Order Constant. Assuming that two separate reactions, a pseudo-first-order reaction (with titanium(III) citrate, rate constant k_1') and a zero-order reaction (with reduced B_{12}, rate constant k_0) proceed simultaneously (see Results and Discussion), the observed disappearance of CCl_4 is given by

$$-\frac{d[CCl_4]}{dt} = k_1'[CCl_4] + k_0 \qquad (1)$$

At $t = 0$, $[CCl_4] = [CCl_4]_0$ = the initial concentration of CCl_4. Equation 1 can be solved for $[CCl_4]$:

$$[CCl_4] = [CCl_4]_0 e^{-k_1' t} - \left(\frac{k_0}{k_1'}\right)(1 - e^{-k_1' t}) \qquad (2)$$

Rearranging eq 2 gives an expression for k_0:

$$k_0 = \frac{k_1'([CCl_4]_0 e^{-k_1' t} - [CCl_4])}{(1 - e^{-k_1' t})} \qquad (3)$$

$[CCl_4]_0$ and k_1' can be evaluated independently from the control (in the absence of titanium(III)) and the blank experiment (only titanium(III) present, no B_{12}), respectively. At each time point t, a $[CCl_4]$ was measured and a k_0 was calculated using eq 3. The k_0 values obtained from eq 3 were then used to calculate the rate constant for the rate-limiting step (see Results and Discussion).

Isotopic Chloroform Analysis. To distinguish the two pathways for chloroform formation (reduction followed by protonation and hydrogen atom abstraction, see Discussion), experiments were conducted in deuterated water (D_2O, 99.9%, Isotec, Miamisburg, OH) in 9-mL amber screw-cap vials following procedures similar to those described in the Kinetic Study section. Different amounts of sucrose were added to the reagent mixture as an additional organic hydrogen source (to encourage the hydrogen abstraction reaction), and the final liquid volume was adjusted to 7.5 mL. Sucrose was chosen as the hydrogen source because (i) it is freely soluble in water, (ii) it does not significantly affect the pH even at high concentrations, (iii) it has negligible effect on ionic strength, and (iv) it does not interfere with the vapor phase GC analysis as some alcohols do. Upon complete transformation of CCl_4 (approximately 4 h), head space samples were analyzed with an HP 5890 GC coupled with an HP 5970 mass selective detector (GC/MSD) and equipped with a 30-m DB-5 fused silica capillary column (0.25 μm film thickness, J & W Scientific). The relative yields of $CDCl_3$ and $CHCl_3$ were obtained by integrating the areas of the ion chromatogram peaks for m/z 84 ($^{12}C^2D^{35}Cl_2$) and m/z 83 ($^{12}C^1H^{35}Cl_2$), respectively. The relative importance of the two chloroform formation pathways was evaluated based on the two peak areas followed by a correction procedure described below to account for the introduction of hydroxyl-H^+ of sucrose, which in effect lowers the purity of deuterium in the medium.

We assume that proton exchange between the hydroxyl groups of sucrose and water was rapid; thus, the percentage of H^+ at each proton-exchange site (of either sucrose or water) was equal to the overall ratio of $[H^+]/[H^+ + D^+]$. This ratio was attained as a function of sucrose concentration by measuring the weights of sucrose and D_2O during solution preparation. For each sucrose concentration, the amount of chloroform formed via protonation was taken as $[CDCl_3]_{prot}$, the total $CDCl_3$ measured, plus $[CHCl_3]_{prot}$, the amount of $CHCl_3$ formed due to protonation with H_2O, HDO, and the −OH of sucrose. $[CHCl_3]_{prot}$ was obtained from $([H^+]/[D^+])([CDCl_3])_{prot}$. The amount of chloroform formed via hydrogen abstraction was then obtained by subtracting $[CHCl_3]_{prot}$ from the total amount of $CHCl_3$ measured ($[CHCl_3]_{tot}$). The corrected $CDCl_3$ area ($[CDCl_3]_{prot} + [CHCl_3]_{prot}$) and $CHCl_3$ area ($[CHCl_3]_{tot} - [CHCl_3]_{prot}$) were used to evaluate the relative significance of the two chloroform formation pathways at different sucrose contents.

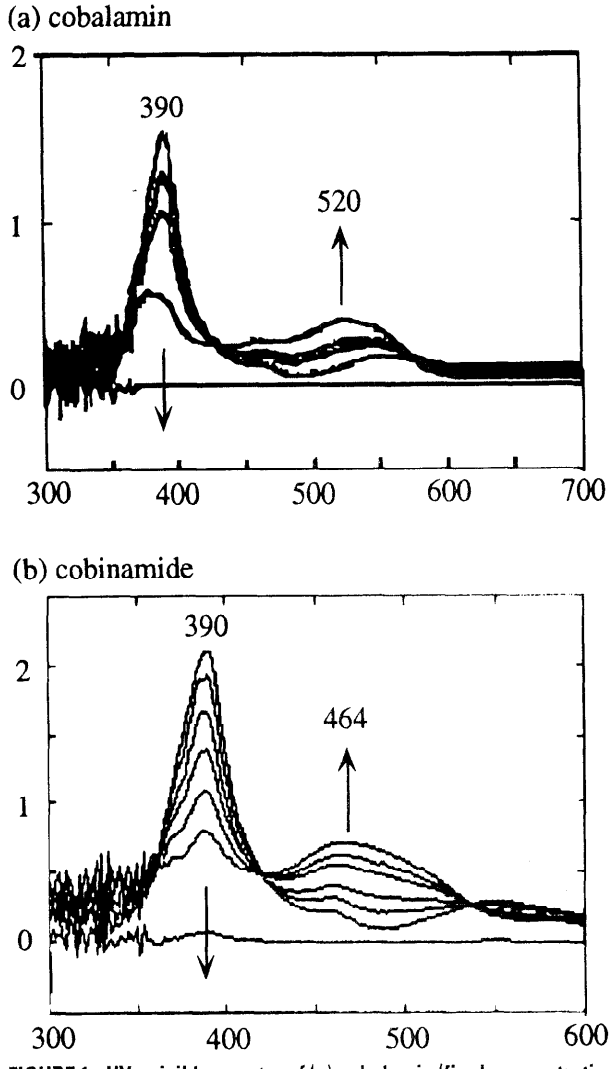

FIGURE 1. UV–visible spectra of (a) cobalamin (final concentration = 50 μM) and (b) cobinamide (67 μM) in titanium(III) citrate solution before and upon addition of increasing amounts of CCl_4. [Titanium(III)] = 6.0 mM; pH 8.2 (0.2 M Tris-HCl); T = 22 °C. The spectra were referenced to blank titanium(III) citrate solution of identical concentration and pH.

UV–Visible Spectra. Using an HP 8451A diode array spectrophotometer, cob(I)alamin and cob(I)inamide were identified by the strong absorption peak at 390 nm (*10, 23, 42*) as vitamin B_{12} (50 μM) or dicyanocobinamide (67 μM) was added anaerobically to 6.0 mM titanium(III) citrate solution at pH 7.3, 8.3, 9.2, and 10.3. The halocarbon–cobalt bonds in alkylcobinamide and cobalamin were indicated by the peak at approximately 464 (*10, 25, 43*) and 520 nm (*29, 44, 45*), respectively.

CO Analysis. Upon complete transformation of CCl_4, head space samples were analyzed on an RGD2 reduction gas detector (Trace Analytical, Menlo Park, CA) for carbon monoxide (CO). CO yield was estimated from the difference in CO peak heights between the experimental and the control (no CCl_4 added). CO calibration standards were purchased from Scott Specialty Gases (San Bernardino, CA).

Results

Reaction of Vitamin B_{12}. *Spectroscopic Data.* In addition to vitamin B_{12}, cyanocobinamide was used to spectroscopically confirm the formation of the alkylcobalt intermediate (*10, 25*). The results are shown in Figure 1. When B_{12} or cobinamide was added to titanium(III) citrate solution

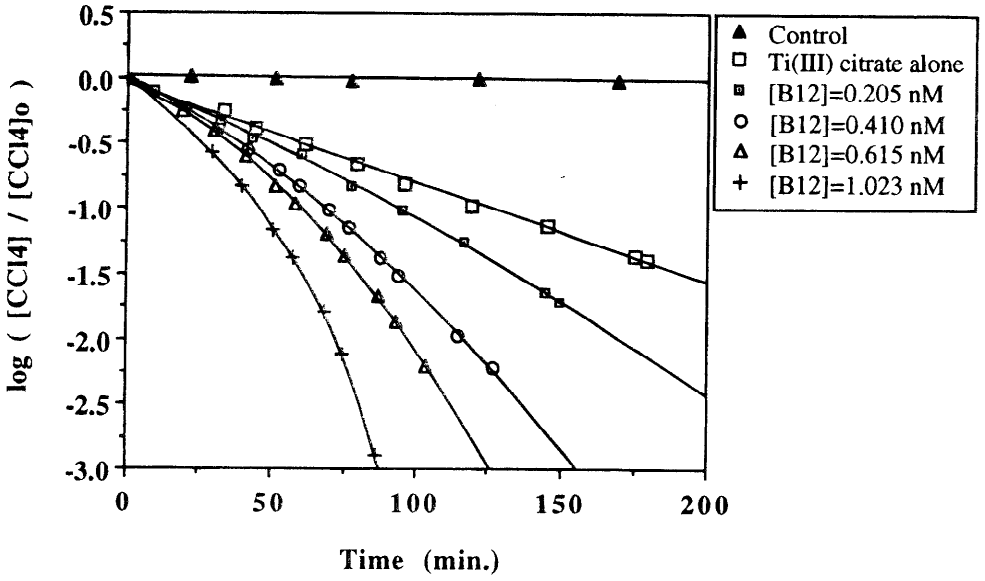

FIGURE 2. Pseudo-first-order reaction curve of CCl_4 with titanium(III) citrate alone and mixed-order reaction curves when mediated by vitamin B_{12}. As $[B_{12}]$ increased, the reaction curve digressed progressively from linearity. [Titanium(III)] = 1.25 mM; $[CCl_4]_0$ = 200 nM; pH 8.12 (0.2 M Tris-HCl); T = 22 °C.

at pH from 7.3 to 10.3, the prominent peak at 390 nm (referenced to titanium(III) citrate solutions of identical concentration and pH; 386 nm if referenced to Milli-Q water) appeared and reached the maximum peak height instantaneously, indicating rapid reduction of Co(III) to Co(I). At pH <6, however, the spectra indicated that Co(II) complexes rather than Co(I) complexes were formed. Upon the addition of CCl_4 to a titanium(III) citrate solution containing cobinamide, the disappearance of the Co(I) and the formation of the cobalt–carbon bond were indicated by the diminishing peak at 390 nm and the concomitant appearance of a broad peak at approximately 464 nm, respectively, and the reaction appeared to be immediate. Similar changes were observed in the case of vitamin B_{12} except that a broad peak appeared at about 520 nm instead.

Because the reduction potential of the B_{12r}/B_{12s} couple (*24*, *46*) is lower than that of the B_{12}/B_{12s} couple (*26*), the ability of titanium(III) citrate to reduce B_{12r} to B_{12s} was examined. B_{12r} was completely and rapidly reduced to B_{12s} in the pH range 7.3–10.3. Thus, should any B_{12r} be generated in the catalytic cycle, Ti(III) reduces it instantly by B_{12s}. Taken together, the spectroscopic data suggest that in neutral and alkaline titanium(III) citrate solution (i) B_{12s} is both the predominant form and the reacting species and (ii) B_{12s} reacts instantaneously with CCl_4 to yield a product or stable intermediate which contains Co–C bond.

Kinetic Data. As shown in Figure 2, the reaction of CCl_4 with titanium(III) citrate in the absence of B_{12} follows pseudo-first-order kinetics (k_1'). With increasing $[B_{12}]$, the rate increased and the reaction progressively deviated from first-order (linear) behavior; at high $[B_{12}]$, where the background reaction with titanium(III) alone became less significant, the kinetics was nearly zero-order (straight lines on a normal–normal plot). k_0, the zero-order constant of the B_{12}-mediated CCl_4 transformation, was estimated from the mixed-order system using eq 3. The initial k_0 values calculated in the course of a reaction were constant for a fixed $[B_{12}]$ and pH, consistent with zero-order kinetics for the B_{12}-catalyzed transformation of CCl_4. This finding contrasts the first-order behavior previously observed under different experimental conditions (*10*). As shown in Figure 3, when $[B_{12}]$ increased, k_0 also increased linearly, indicating first-order dependence of k_0 on $[B_{12}]$. The slope k_3' (= $k_0/[B_{12}]$), which represents the pseudo-first-order rate constant, increased by a factor of $>10^2$ from pH 7.3 to pH 10.3 (see Figure 4).

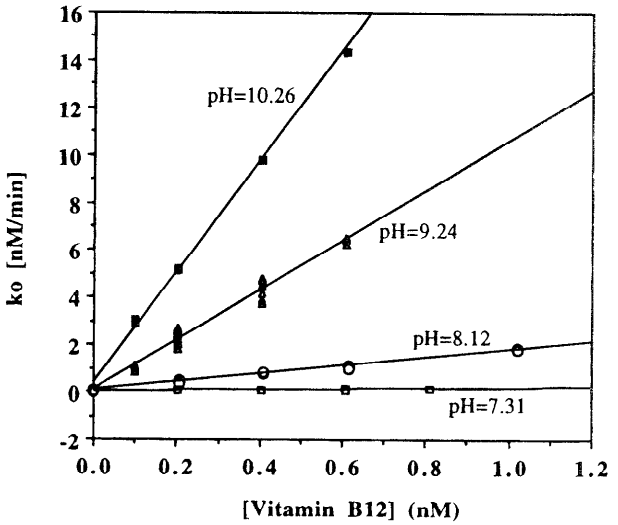

FIGURE 3. Zero-order rate constant (k_0) as a function of cobalamin concentration and pH. Note that at each $[B_{12}]$ and pH, the multiple data points collected superimpose, indicating the constancy of k_0. The slopes correspond to the pseudo-first-order rate constants ($k_3' = k_0/[B_{12}]$) of the rate-limiting step (rls) at different pH. [Titanium(III)] = 1.25 mM; $[CCl_4]_0$ = 200 nM; T = 22 °C.

The effect of [Ti(III)] on k_0 was also investigated. At neutral pH, [Ti(III)] had little effect on k_0 at constant $[B_{12}]$, and the rate law was $R = k_3'[CCl_4]^0[B_{12}]^1$. At pH 9.8, however, k_0 increased in proportion to [Ti(III)] (Figure 5). The results suggested predominance of a different mechanism and first-order involvement of titanium(III) in the rate-limiting step. Incorporating titanium(III) into the above rate law gives $R = k_3[CCl_4]^0[B_{12}]^1[Ti(III)]^1$. The second-order rate constant, k_3 (= $k_0/[B_{12}]/$[titanium(III)]), at pH 9.8 was 340 ± 40 M^{-1} s^{-1}.

Reaction with Hematin. Addition of hematin also accelerated the degradation rate of CCl_4, but unlike the

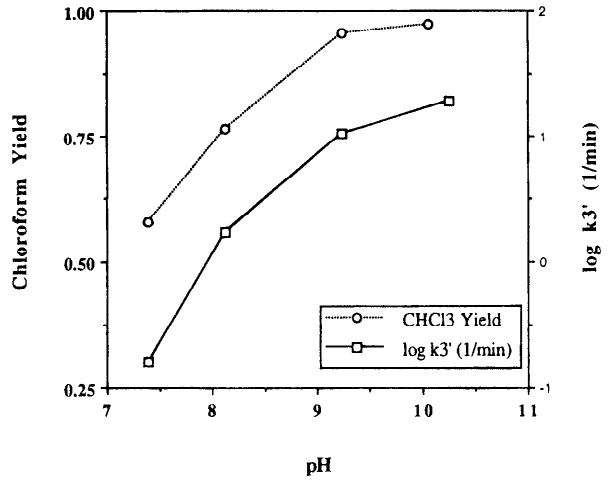

FIGURE 4. pH dependence of the pseudo-first-order rate constant (k_3') and $CHCl_3$ yield. [Titanium(III)] = 1.25 mM; $[CCl_4]_0$ = 200 nM; $[B_{12}]$ = 0.1–1.0 nM; T = 22 °C.

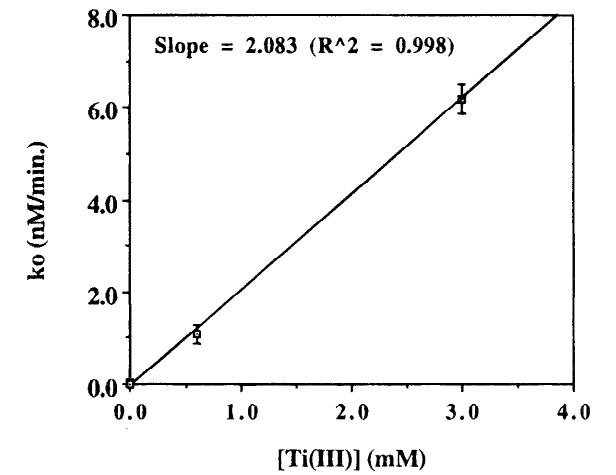

FIGURE 5. Dependence of CCl_4 transformation rate on titanium(III) concentration. pH 9.8 (0.2 M HCO_3^-/CO_3^{2-}); $[B_{12}]$ = 0.1 nM; T = 22 °C.

vitamin B_{12} system, only pseudo-first-order kinetics was observed up to pH 10.8 both in the presence and absence of hematin. Above pH 11, acquisition of kinetic data was precluded by the formation of a deep blue precipitate (presumably titanium(III) hydroxide). The rate constants k_1' in the presence and absence of hematin exhibited strong and similar pH dependence, as shown in Figure 6. Hematin was not as effective a catalyst as vitamin B_{12}, and higher molar concentrations (approximately 1 order of magnitude) were required to achieve similar CCl_4 transformation rate. In contrast to B_{12}, hematin was found to be deactivated fairly rapidly. After a certain number of turnovers, little catalytic activity of hematin was observed following re-spiking of CCl_4, and the reaction rate was essentially the same as the background rate (i.e., reaction with titanium-(III) citrate). The turnover number estimated at pH 8.0 using both methods (see Kinetic Study) was approximately 27, similar to the value (26) previously reported for cytochrome P_{450} enzyme at pH 7.4 (*47*). The same procedures at pH 9.9 gave a higher value of 42 (Figure 7).

The strong binding of CO to Fe(II) porphyrins has been well-established and was considered a possible cause of heme deactivation. Nearly complete inactivation of heme in the reduction of CCl_4 was observed when the system was presaturated with CO. However, the amount of CO measured (see Product Formation) was too low to entirely

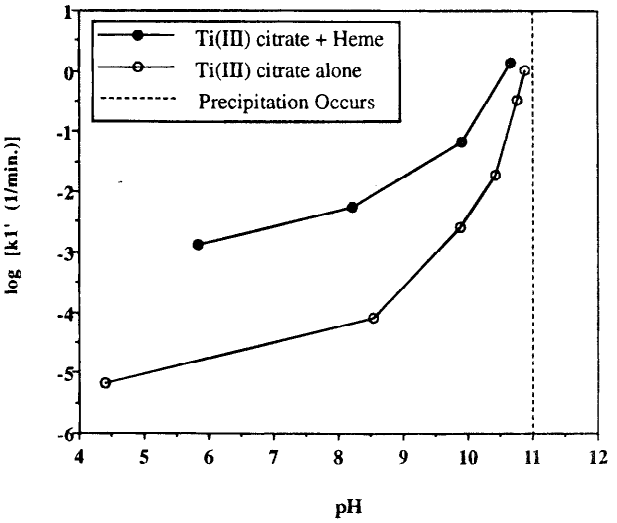

FIGURE 6. Pseudo-first-order rate constant (k_1') as a function of pH in titanium(III) citrate solution in the presence and absence of hematin. [Titanium(III)] = 4.8 mM; $[CCl_4]_0$ = 1.0 μM; [hematin] = 15.5 nM; T = 22 °C.

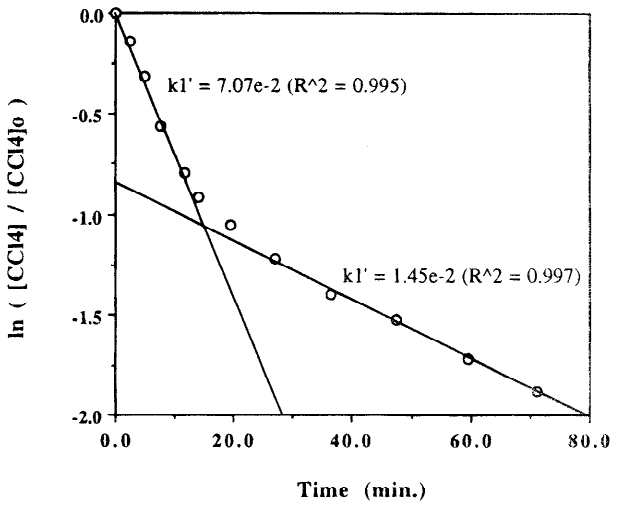

FIGURE 7. Deactivation of hematin during reaction with CCl_4. The abrupt change in slope (k_1') indicates when hematin was completely deactivated. [Titanium(III)] = 4.86 mM; [hematin] = 15.5 nM; $[CCl_4]_0$ = 1.1 μM; T = 22 °C; pH 9.90 (0.2 M HCO_3^-/CO_3^{2-}). The turnover number calculated at this pH is approximately 42 (CCl_4 molecules transformed/hematin molecule deactivated), somewhat higher than the value (27) obtained at pH 8.0 (0.2 M Tris-HCl).

account for the observed inactivation and other mechanisms must be involved.

Product Formation. Chloroform was the major dechlorination product observed in the pH range studied in both coenzyme systems. Under the experimental conditions, the yield was found to increase with pH, [titanium(III)], and organic material content in the solution and was independent of whether a coenzyme was added or which coenzyme was used. $CHCl_3$ yield increased from about 58% at pH 7.3 to greater than 95% at pH 10.3 (Figure 4). With increasing [titanium(III)] at constant pH, $CHCl_3$ production also increased, although not in proportion (data not shown).

Experiments conducted in D_2O solution containing catalytic amount of hematin or B_{12} and different concentrations of sucrose have demonstrated that total chloroform yield also increased with sucrose concentration, as shown in Figure 8 for hematin. In the absence of sucrose, $CDCl_3$ was the exclusive product, and very little $CHCl_3$ was formed.

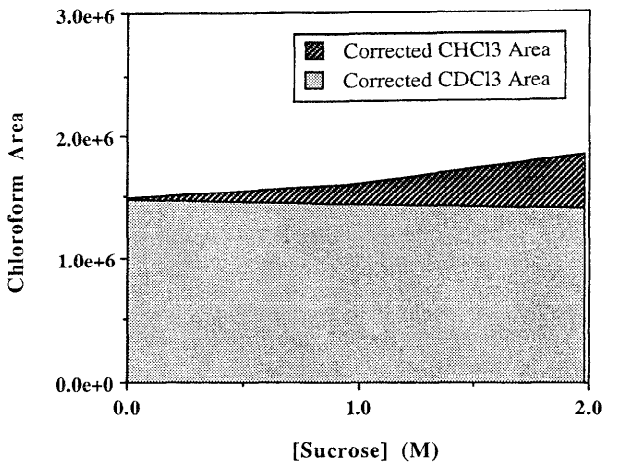

FIGURE 8. Corrected $CHCl_3$ and $CDCl_3$ yields (reported as $CH^{35}Cl_2$ and $CD^{35}Cl_2$ ion chromatogram peak areas) at different sucrose concentrations: pH 7.8 (0.2 M Tris-HCl buffer); [titanium(III)] = 5.0 mM; $[CCl_4]_0$ = 28 μM; [hematin] = 0.35 μM; T = 22 °C.

This suggests that the combination of $:CCl_3^-$ and D^+ was the predominant mechanism. As we increased the sucrose content, $CHCl_3$ yield increased accordingly, partly at the expense of $CDCl_3$. The production of $CHCl_3$ indicates the incorporation of covalent hydrogen from sucrose by hydrogen abstraction. The amounts of $CDCl_3$ and $CHCl_3$ (after correction, see Isotopic Chloroform Analysis) may then be used to evaluate the relative importance of the two chloroform formation pathways, protonation of $:CCl_3^-$ and hydrogen abstraction by $^{\bullet}CCl_3$. Proton exchange between water and $CHCl_3$ was excluded as a confounding factor since no deuterated chloroform was found in pH-buffered D_2O over the same incubation time (except at pH 10, where 25% of total chloroform underwent proton exchange).

A small amount of CO was detected in the head space samples in both coenzyme systems. CO was previously detected as a minor CCl_4 transformation product in a similar system containing cobalamin (*27, 48*) and during anaerobic incubation of CCl_4 with reduced cytochrome P_{450} hemoprotein (*34, 47, 49, 50*). CO has been known to form from CCl_4 (and other tetrahalomethanes (*27*)) via hydrolysis of dichlorocarbene, $:CCl_2$.

Discussion

Vitamin B_{12} System. Our spectral data are consistent with the hypothesis that in neutral and basic titanium(III) citrate medium B_{12s} is the predominant and reacting species and that, when CCl_4 was added, a product or stable intermediate containing a Co–C bond was formed as a result of the reaction of B_{12s} with CCl_4. B_{12s} is known for its strong nucleophilicity in the axial direction (*19, 51, 52*), which involves the lone electron pair in the antibonding d_z2^* orbital of Co(I). The reaction of B_{12s} with halogenated alkanes via the S_N2 (also known as "oxidative addition") mechanism has been established (*19, 22, 53*). This oxidative addition of B_{12s} proceeds rapidly (*19*) by the attack of the lone pair at the electropositive carbon center to give an alkylcobalt complex. Like conventional S_N2 reactions, this reaction is accompanied by inversion of configuration (*19, 54*) and is possibly reversible to a small extent (*53*). In the case of CCl_4, B_{12s} attack gave rise to trichloromethylcobalamin (CCl_3-Cbl), the intermediate that contained a Co–C bond. Although the bulky chlorine atoms can exert significant steric strain (*44*), CCl_3-Cbl is known to be moderately stable (*31, 45*), and therefore the decomposition of CCl_3-Cbl is expected to be the rate-limiting step. The pseudo-first-order constant k_3' (= $k_0/[B_{12}]$) in Figures 3 and 4 thus represents the decomposition rate of CCl_3-Cbl. At pH 7.3, the decomposition of CCl_3-Cbl proceeded slowly via a mechanism which did not involve Ti(III). This mechanism is hypothesized to be sterically induced, spontaneous Co–C bond homolysis, similar to other sterically strained alkylcobalamins (*19, 55*). Penley et al. (*44*) observed the steric effect when more than one Cl atom is bonded to the α-carbon (no such effect was observed in the case of fluorine) and suggested that the steric strain may lengthen the Co–C bond and facilitate bond homolysis. The half-life of CCl_3-Cbl measured at pH 7.3 was 4.5 min, which was comparable to the reported half-lives of several sterically hindered secondary alkylcobalamins (*55*). For example, the half-life of isopropylcobalamin is 3.0 min at pH 7.0 at room temperature.

This reaction scheme is in accord with the observed spectroscopic and kinetic data. Since B_{12s} reacted instantaneously with CCl_4, essentially all B_{12} existed as CCl_3-Cbl, and the degradation rate of CCl_4 was limited by the decomposition of CCl_3-Cbl when $[CCl_4] \gg [B_{12}]$. k_0 was therefore independent of $[CCl_4]$ and first-order with respect to $[B_{12}]$ (= $[CCl_3$-Cbl$]$). These results suggest that the system can be described by a mechanism analogous to the Michaelis–Menten model in the zero-order regime. The coenzymatic reaction occurs via a reversible combination of the coenzyme, C (B_{12s}), and the substrate, S (CCl_4), to form an intermediate CS* (CCl_3-Cbl), which decomposes to yield the product and regenerate a free coenzyme:

$$C + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CS^* \xrightarrow[\text{(rls)}]{k_3'} C + \text{product} \qquad (4)$$

When $[C] \ll [S]$ and C has a strong affinity for S (as in the case of B_{12s} and CCl_4) so that all C is present as CS* (i.e., $[C]_{tot} \approx [CS^*]$), then the system is saturated, K_s (saturation constant) $\ll [S]$, and the substrate transformation rate (R) approaches the maximum value, R_{max}:

$$R = \frac{k'_3[CS^*][S]}{K_s + [S]} \approx k'_3[CS^*] \approx k'_3[C]_{tot} = R_{max} = k_0 \quad (5)$$

R_{max} is independent of [S] and first-order in $[C]_{tot}$, as observed in the CCl_4-B_{12} system.

The strong pH effect on k_3' and the first-order dependence of k_3' on [titanium(III)] at pH 9.8 implied that a mechanism other than steric hindrance might be responsible for the decomposition of CCl_3-Cbl, in which titanium(III) was involved. Halomethyl–Co complexes are known to be alkali-labile (*56*); however, the pH dependence of the CCl_4 transformation rate (i.e., Co–C bond cleavage rate) cannot be explained simply by hydrolysis of CCl_3-Cbl because (i) hydrolysis does not account for the increasing $CHCl_3$ yield with pH (FIgure 4) or the rate dependence on [titanium(III)] (Figure 5), and (ii) hydrolysis of halomethylcobalamins proceeds only under rather extreme conditions (e.g., heating at pH 14). One hypothesis is the one-electron reduction of CCl_3-Cbl by titanium(III) to a radical anion, $[CCl_3\text{-Cbl}]^{\bullet -}$, as suggested by Krone et al. (*25*). Scheffold et al. (*57*) have provided evidence for this hypothesis. They pointed out that alkylcobalamins with

electron-withdrawing substituents (such as halogens) at the α-carbon are more easily reduced. Moreover, Zhou et al. (*58*) stated that increasing the degree of substitution on the α-carbon of an alkylcobalamin can shift its reduction potential to more positive values. Nonetheless, the E° value of titanium(III) citrate seems too high to accomplish such reduction. For example, the E° for methylcobalamin (CH_3-Cbl) is −1.57 V vs SCE (or −1.33 V vs SHE) in neutral solution (*59*) and that for the fluorinated analogue, CF_3-Cbl, is approximately −1.26 V vs SCE (*59, 60*) or −1.04 V vs SHE. Since chlorine is less electronegative than fluorine, the E° of CCl_3-Cbl would be expected to be more negative than that of CF_3-Cbl and therefore unlikely to be reduced by titanium(III) citrate (note: higher E° is possible if the organic substituent can provide a resonance structure, as in the case of (methoxycarbonyl)methylcobalamin; however, such an effect is lacking in trihalomethylcobalamins). On the basis of this hypothesis, the E° of CCl_3-Cbl must have been shifted to a value less negative than that of CF_3-Cbl due to the steric encumbrance resulting from bulky Cl atoms. In addition, since [Ti(III)] ≫ [Ti(IV)] in the medium, the actual reduction potential of titanium(III) citrate was lower than the standard value, which would also favor the reduction of CCl_3-Cbl. Since we failed to observe [titanium(III)] dependence of k_3' at pH 7.3, the redox potential of titanium(III) citrate at neutral pH is presumably too high to reduce CCl_3-Cbl. Assaf-Anid et al. (*29*) proposed the formation of $[CCl_3\text{-Cbl}]^{\bullet -}$ in a system redox-buffered by dithiothreitol (DTT). Their proposal contradicts our finding since, in the pH range studied, the E° values of DTT are higher than that of titanium(III) citrate at neutral pH.

Alkylcobalamin radical anions are highly unstable and decompose immediately because the Co−C bond order is reduced from 1 to 0.5 upon addition of an antibonding d_{z^2}* electron (*58, 61, 62*). Homolytic decomposition of both CCl_3-Cbl and $[CCl_3\text{-Cbl}]^{\bullet -}$ yields trichloromethyl radical, $^\bullet CCl_3$. We propose that in titanium(III) citrate medium at pH > 7, $^\bullet CCl_3$ is mostly reduced by titanium(III) citrate medium at pH > 7, $^\bullet CCl_3$ is mostly reduced by titanium(III) to trichloromethyl carbanion, $:CCl_3^-$, which rapidly abstracts a proton from water to form $CHCl_3$. This postulation is supported by (i) the increase in chloroform yield with increasing [titanium(III)] and pH and (ii) the finding that $:CCl_3^-$ was the exclusive precursor for chloroform at low organic content. To a small extent, $:CCl_3^-$ might eliminate a Cl^- to give dichlorocarbene, $:CCl_2$, which then hydrolyzed to yield CO. Although heterolysis of the radical anion to form $:CCl_3^-$ and B_{12r} has also been suggested to occur (*25, 58*), our data indicate that homolysis is probably more significant.

Figure 9 illustrates the proposed B_{12}-catalyzed CCl_4 transformation pathways in titanium(III) citrate solution at neutral and alkaline pH. Titanium(III) appears to be involved in both the rate-limiting reductive decomposition of CCl_3-Cbl (k_3) at high pH and the reduction of $^\bullet CCl_3$ (k_5) to yield $CHCl_3$. Figure 4 shows the reaction rate and the chloroform yield exhibit similar pH dependence. The change in redox potential of titanium(III) citrate with pH probably resulted from the formation of mixed citrate–hydroxy complexes, as suggested for titanium(IV) citrate (*63*). A color change from red to greenish brown to blue was observed as pH increased from 7.3 to 10.3, possibly due to partial substitution of hydroxide for citrate.

The previous model (eqs 4 and 5) can be modified to account for the first-order involvement of titanium(III) in

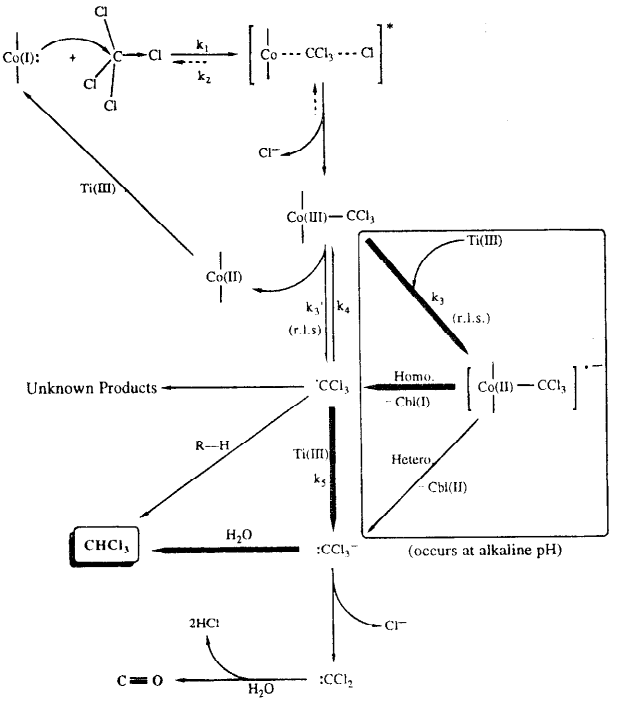

FIGURE 9. Proposed CCl_4 transformation pathway in titanium(III) citrate solution containing catalytic amount of cyanocobalamin: Cbl = cobalamin.

the rate-limiting step (rls) at alkaline pH:

$$C + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CS^* \tag{6}$$

$$CS^* + Ti(III) \xrightarrow[(rls)]{k_3} [CS^*]^- + Ti(IV) \xrightarrow[-Ti(IV)]{fast} C + product \tag{7}$$

$$R \approx k'_3[C]_{tot} = k_3[C]_{tot}[Ti(III)] = R_{max} = k_0 \tag{8}$$

Hematin System. The reduction mechanism of organic halides by heme and hemoproteins has been described by Wade and Castro (*11, 12, 30*) to be an innersphere electron transfer process. The first step of this process involves a reversible complexation of CCl_4 to the axial site of heme, yielding a Cl-bridged transition state complex. The decomposition of this complex via C−Cl bond cleavage to give $^\bullet CCl_3$, the same reactive intermediate generated in the B_{12} system, was proposed to be rate-limiting. Again, we hypothesize that in titanium(III) citrate solution, $^\bullet CCl_3$ was mainly reduced to $:CCl_3^-$, which then accepted a proton from water; consequently, the same chloroform yield was obtained with both cofactors at the same pH and [Ti(III)].

$^\bullet CCl_3$ was also produced by the hemoprotein cytochrome P_{450} during anaerobic incubation with CCl_4 and has been known to initiate lipid peroxidation, membrane damage, and deactivation of the enzyme (*47, 49, 64, 65*). The result shown in Figure 8 indicates that hydrogen abstraction by $^\bullet CCl_3$ may become significant as organic content increases. This finding is in accordance with the observation by Ahr et al. (*49*), who reported that >90% of the hydrogen in chloroform generated during incubation of CCl_4 with NADPH-reduced cytochrome P_{450} was derived from organic material. When generated at the heme site of P_{450}, $^\bullet CCl_3$ most likely abstracts a hydrogen atom from the immediate apoprotein matrix before it diffuses into the solution where it could be reduced by the bulk reductant or by another

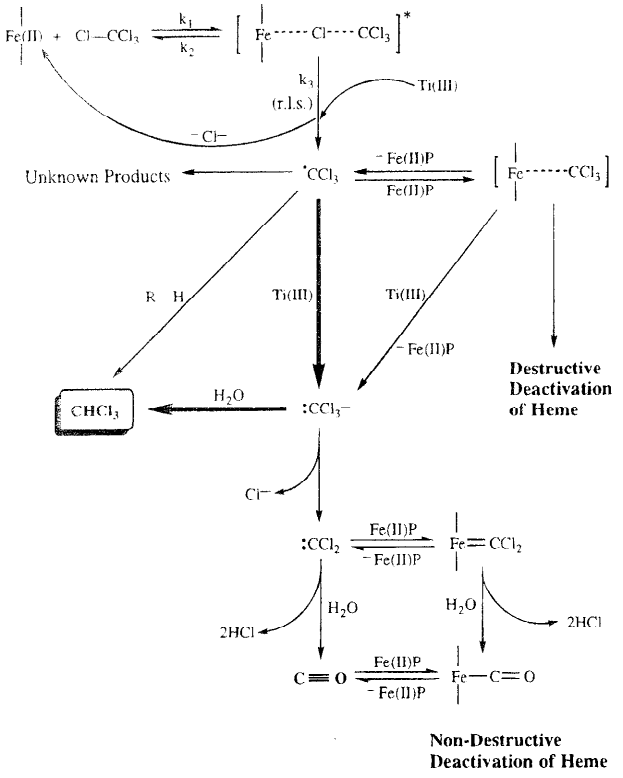

FIGURE 10. Proposed CCl_4 transformation pathway in titanium(III) citrate solution containing hematin: Fe(II)P = iron(II) protoporphyrin IX.

reduced P_{450} enzyme. In addition, NADPH is not as strong a reductant as titanium(III) and thus may not reduce $\cdot CCl_3$ to $:CCl_3^-$ as effectively. The quantitative $CHCl_3$ yield reported by Castro et al. (*35*) from reaction of CCl_4 with bare heme in an organic cosolvent also suggests that ·H abstraction could be predominant at high organic contents. The increasing *total* chloroform production with increasing sucrose concentration shown in Figure 8 indicates that when the system contained little organic matter, a portion of $\cdot CCl_3$ not scavenged by titanium(III) was consumed via unknown pathways.

In our system, iron(II) porphyrin was deactivated during reaction with CCl_4. Previously, deactivation of heme and hemoprotein by CCl_4 was reported (*47, 64–66*) and was suggested to involve reactive metabolites $\cdot CCl_3$ and $:CCl_2$ (*50*). Heme has been recognized to be the site and target of such attack (*47, 50, 64*), and the porphyrin moiety was irreversibly modified (*47, 66–69*). The observation that the addition of a radical scavenger suppressed heme loss (*65, 70*) suggests that this process is initiated by free $\cdot CCl_3$. Furthermore, the finding that $\cdot CCl_3$ attacks heme but not iron-free protoporphyrin (*66, 71*) indicates the intramolecular nature of the deactivation process. It is likely that $\cdot CCl_3$ rapidly (*72*) forms a complex with a heme first and then disrupts the porphyrin structure of that particular heme molecule, presumably by binding covalently to the porphyrin (*70, 73*). The proposed reductive transformation scheme for CCl_4 in titanium(III) citrate solution containing hematin is shown in Figure 10. The greater turnover number at higher pH can be explained by the proposed scheme: as pH increases, titanium(III) citrate becomes more reducing and thus can prevent heme deactivation by scavenging more $\cdot CCl_3$ (and producing more $CHCl_3$). Dichlorocarbene, another metabolite suggested to cause heme loss (*50, 74*), was shown to be not significantly involved in heme deactivation (*47*).

The formation of $:CCl_2$ (*75, 76*) in organic iron porphyrin solution and CO (*34, 47, 49, 50*) in neutral aqueous medium containing hemoprotein in the presence of an excess reductant has been widely reported. $:CCl_2$ was further confirmed by a carbene-trapping agent (*77*). In a separate study using hematin as a catalyst and cysteine as a reducing agent in an aqueous medium, CO yields of up to 16% were measured (data not shown). The mechanism of chloroform hydrolysis by OH^- proposed (*35*) to account for $:CCl_2$ and CO formation was unable to explain our results because (i) base hydrolysis was not observed (except at pH 10 to a small extent), and no CO was detected at pH up to 10.5 when $CHCl_3$ was the substrate (concentration the same as $[CCl_4]_0$ used in our experiments); and (ii) CO yield in the hematin-mediated system did not always increase with pH. Production of $:CCl_2$ is possible in a reaction involving $:CCl_3^-$ as an intermediate.

Conclusions

The reaction schemes of CCl_4 transformation by vitamin B_{12} or hematin in aqueous titanium(III) citrate medium are proposed. Spectroscopic and kinetic data as well as data from isotopic studies are all in agreement with these proposed mechanisms. B_{12s} was the reacting species and attacked CCl_4 via S_N2 pathway, giving rise to the stable intermediate CCl_3-Cbl. The overall reaction rate appeared to be governed primarily by the stability of CCl_3-Cbl and the reducing power of titanium(III). CCl_3-Cbl decomposed either spontaneously or by reacting with titanium(III) at alkaline pH, presumably via electron transfer and the formation of a transient radical anion. The rate constant was zero-order in CCl_4, first-order in B_{12}, and first-order in titanium(III) at high pH. Hematin was not only a less efficient catalyst than B_{12} but also more susceptible to deactivation induced by $\cdot CCl_3$ or CO. $\cdot CCl_3$ was presumably the primary intermediate generated in the rate-limiting steps with both coenzymes; it was then transformed to $CHCl_3$ by either accepting an electron from titanium(III) and a proton from water or abstracting ·H from organic material.

Our data have shown that the concentration and the reducing power of the reducing agent can strongly influence both CCl_4 transformation rate and chloroform yield. The catalysts, vitamin B_{12} and hematin, only impact the reaction rate. The rate and the product distribution are also affected by the type of reducing agent. For instance, thio reducing agents generate B_{12r} which, unlike B_{12s}, reacts with CCl_4 via a radical-type mechanism. Preliminary experiments conducted in our laboratory with thio reductants (such as cysteine or DTT) and vitamin B_{12} exhibited slower reaction rates, a different pH dependence, and lower $CHCl_3$ yields. Specifically, with cysteine as the reducing agent, the $CHCl_3$ yield *decreased* with increasing pH in the range of 8–12 (data not shown). Taken together, our results indicate that reducing agents play a significant role in such coenzymatic transformation and that results obtained in one reductant system cannot be extrapolated to systems where a different type of reducing agent is employed.

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